CHAPTER 1

INTRODUCTION

1.1 The genus *Shigella*

The prototypical organism, *Shigella dysenteriae* type 1, was first isolated and later described by Kiyoshi Shiga in his publication in 1898 (Keusch, 2002). He had originally named the organism *Bacillus dysenteriae* though it was almost always referred to as Shiga's bacillus. Ultimately, the genus name *Shigella* was proposed and adapted to honour the discoverer.

Shigella spp. are slender, non-motile, Gram-negative bacilli that conform to the definition of the family Enterobacteriaceae (Bopp *et al.*, 1999). The genus is made up of four serogroups that historically have been treated as species: serogroup A as *S. dysenteriae* (13 serotypes), serogroup B as *S. flexneri* (6 serotypes with a total of 15 sub-types), serogroup C as *S. boydii* (18 serotypes) and serogroup D as *S. sonnei* (1 serotype) (Keusch, 2002). Serogrouping is carried out on the basis of common O-specific polysaccharide antigens that separate the species.

The four *Shigella* species are considered to be members of a single genus with *Escherichia coli* because they share similarities in more than 90% of their genetic makeup (Maurelli *et al.*, 1998). A recent report by Pupo *et al.* (2000) maintained that *Shigella* had emerged from multiple independent origins of *E. coli* 35 000 to 270 000 years ago, predating the development of agriculture, and making shigellosis one of the early infectious diseases of humans. Gene transfer by conjugation and transduction, and formation of recombinants between *Shigella* and *E. coli* occur with high frequency due to colinearity of their chromosomes. The enteroinvasive *E. coli* (EIEC) is often confused with *Shigella*, and vice-versa, as EIEC carry plasmids homological to the large virulence or invasive plasmid of *Shigella*, and both the organisms are responsible for a clinically similar diarrhoeal disease.

Most studies on the molecular pathogenesis of *Shigella* are performed in *S. flexneri* 2a and 5a. Venkatesan *et al.* (2001) had been successful in determining the complete DNA sequence and analysis of the large virulence plasmid (210 kb) in *S. flexneri* 5a. It had 286 open reading frames (ORFs), of which 53% were related to known and putative insertion sequence (IS) elements. A predictable consequence of this discovery is the observed pre-inclination of *Shigella* to change its genome frequently and acquire DNA across bacterial species.

The genome of *S. flexneri* 2a, the most prevalent species causing shigellosis in the developing countries, had also been sequenced recently (Jin *et al.*, 2002). It is composed of approximately 4 600 kb of chromosomes and has a large virulence plasmid of 222 kb. The finding of the chromosomes sharing a common "backbone" sequence with those of *E. coli*, strengthens the present day argument for both *Shigella* and *E. coli* to be grouped into the same genus. Most of the 13 translocations and inversions are associated with deletions or acquired DNA sequences, of which there are several potential bacteriophage-transmitted pathogenicity islands (PAIs). With the mapping of the genome, researchers maybe able to understand better the pathogenicity of *Shigella* and consequently, develop novel preventive and treatment strategies against shigellosis.

1.2 Clinical manifestation

Members of the genus *Shigella* are the causative agents of shigellosis or bacillary dysentery. Initially, there is a passing of watery diarrhoea accompanied by fever and abdominal cramps. It may progress to classic dysentery ("bloody diarrhoea") with scant stools containing blood, mucus and pus. *S. flexneri* and *S. dysenteriae* type 1 infections are usually characterized by frequent passings of small amounts of stool and mucus or blood. At times, watery stool followed by typical dysenteric stool maybe

present with *S. dysenteriae* type 1 infection. *S. sonnei* and *S. boydii* infections are less severe with watery stool but little mucus or blood.

The infection is usually self-limited; however when it subsides, the intestinal ulcers heal with scar tissue formation. Uncomplicated recovery is usual and the organisms rarely cause other types of infections. Adversely, in 3 to 50% of cases, depending on the virulence of the strain, the nutritional and immune status of the host, the initial infection maybe followed by neurological complications or kidney failure. Serious complications do occur at greatest frequencies in malnourished infants, toddlers, older adults and immunocompromised individuals (Salyers & Whitt, 1994; Morello *et al.*, 1998; Bopp *et al.*, 1999; Khan *et al.*, 1999).

1.3 Epidemiology

The only known natural reservoirs of *Shigella* are humans and other large primates. Majority of the disease transmission is by person-to-person spread although intake of contaminated food or water can also be the causative factor. Shigellae can be transmitted through sexual intercourse as evident from an increasing occurrence of shigellosis amongst homosexual men (O'Sullivan *et al.*, 2002).

A recent review by WHO (Kotloff *et al.*, 1999) had estimated the annual number of shigellosis to be 164.7 million, of which approximately 99% were reported in developing countries with 1.1 million deaths. Shigellosis-afflicted children under five years of age contributed to 69% of the morbidity and 61% of deaths. Fatality from its infection is extremely rare in the developed countries, but morbidity can be considerable when outbreaks happen in custodial institutions, daycare centers, among soldiers and travelers. Of more important interest, is the latter group which is being infected with multiresistant *Shigella* strains with increasing frequency and giving rise to an estimated 580 000 cases of shigellosis annually.

The majority of *Shigella* species in developing countries are *S. flexneri* followed by *S. sonnei*, *S. boydii* and *S. dysenteriae*. The latter species, infamous for being the most virulent, is seen most often in South Asia and sub-Saharan Africa. *S. sonnei* is the most common species in industrialized countries, with *S. flexneri* being the next most common.

Oubreaks of diarrhoea due to shigellosis are almost endemic in under-developed countries. In Bangladesh, over 185 000 people suffered from acute diarrhoea and 151 had died as a result of it in 1998 (WER, 1998). Sierra Leone reported 3 094 cases of shigellosis caused mainly by *S. flexneri* and *S. dysenteriae* type 1 with 132 deaths in 1999 (WER, 2000). Both strains were found to be sensitive to nalidixic acid and ciprofloxacin. Resistance to antibiotics commonly used for shigellosis treatment was also previously noted in a 1998 outbreak in Cameroon (237 cases with 60 fatalities).

1.4 Pathogenesis and virulence-associated factors

1.4.1 Pathogenesis

Ingestion of as few as 100 to 200 shigellae result in attack rates of 38 to 39% (DuPont *et al.*, 1989); this indicates that the organism survives low pH effectively. The relative resistance of shigellae to gastric acid when compared with *Salmonella* or *E. coli* helps to enhance the survival of small numbers of ingested organisms and provide them the opportunity to infect the intestinal mucosa in the colon (Hale, 1991).

There are mainly three phases involved in the pathogenicity of *Shigella*:

a) an initial cell invasion of the bacteria to gain entry into host cells,

b) the lysis of the endocytic vacuole and release of the ingested bacteria to the cytoplasm, and

c) the intracellular spread of the bacteria to the plasma membrane where they are able to invade into adjacent cells.

In order for *Shigella* to enter an epithelial cell, the bacterium must adhere to its target cell and be internalized by a process similar to phagocytosis via an endosome, which is subsequently lysed to gain access into the cytoplasm (Fig 1.1). Extracellular *Shigella* are nonmotile but intracellular bacteria are able to move to occupy the entire cytoplasm of the infected cell and to spread between cells. After entry into the cell, intracellular movement occurs by the bacterium expressing both the Olm (organelle-like movement) phenotype and the Ics (intercellular spread) phenotype. The Olm phenotype allows the bacterium to "slide" along actin stress cables inside the host cell. The Ics phenotype enables it to "spread". Movement between neighbouring cells is mediated by the formation of protusions at the poles of *Shigella*. Thus, the bacterium can move through the host cell and penetrate into the next cell without coming in contact with the extracellular medium where they would be rendered nonmotile (Salyers & Whitt, 1994; Keusch, 2002).

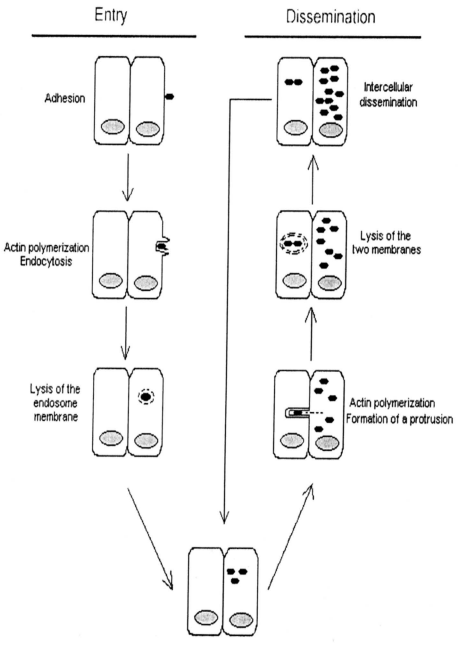


Fig 1.1

Entry and dissemination of *Shigella* in epithelial cells *in vitro*
(adapted from Parsot & Sansonetti, 1996)

1.4.2 Virulence-associated factors

Virulence genes of bacterial pathogens maybe encoded on plasmids, bacteriophages or the chromosome. They are often multifactorial and coordinately regulated, and the genes tend to be clustered in the genome. Many of the virulence-associated genes are restricted to pathogenic organisms and have been introduced into genomes by lateral transfer (Ochman & Moran, 2001). Deletion of genes can also serve as a means of gaining pathogenicity. Introduction of *ompT* (encoding a surface protease) suppressed *Shigella* virulence by disrupting intercellular spread; hence, in *Shigella*, DNA deletion is crucial to the development of virulent strains (Nakata *et al.*, 1993).

1.4.2.1 Plasmid-encoded

The structural genes required for invasion and intercellular spreading of shigellae are encoded within a 31-kb region of a 230-kb virulence plasmid (Dorman & Porter, 1998). Strains with this large plasmid could penetrate tissue culture cells irrespective of the original host of the plasmid (Watanabe & Nakamura, 1985). The plasmid virulence island contains two adjacent loci that are activated in response to environmental cues to encode the effector molecules and the secretory mechanism required for the complete process of invasion.

The *ipa* (invasion plasmid antigen) operon encoding four secreted Ipa proteins, Ipa A-D, makes up the first locus that is also aptly referred to as the invasion-associated locus (*ial*) (Sethabutr *et al.*, 1993). The *ial* locus lies within a 2.5-kb *Hind*III fragment in the large virulence plasmid, near a region that is a hot spot for spontaneous deletions (Sasakawa *et al.*, 1986). It directs epithelial cell penetration by both *Shigella* and EIEC (Frankel *et al.*, 1990). Ipa B-D are the only secretory proteins required for induction of

actin polymerization and bacterial entry (Ménard *et al.*, 1996). Ipa A is not necessary to trigger *Shigella* entry into epithelial cells.

The *ipaH* gene is unique in that there are five complete or partial copies on both the virulence plasmid and the chromosome of *Shigella* and EIEC (Hale, 1991). However, the gene does not lie in or near the *ipaBCD* gene cluster (Venkatesan *et al.*, 1989). *ipaH* is postulated to act by modifying the host response to *Shigella* infection. The protein may inhibit both thrombus formation and the recruitment of inflammatory cells into the lamina propria; thus facilitating the dissemination of shigellae within the mucosa and at the same time, aggravating the loss of blood into the faeces of dysenteric patients (Hale, 1991). Fernandez-Prada *et al.* (2000) confirmed that *S. flexneri* $IpaH_{7.8}$ aids the escape of the bacteria from phagocytic vacuoles in the macrophages.

The second locus is the secretory apparatus controlled by two operons respectively designated *mxi* (membrane expression of invasion plasmid genes) and *spa* (secretion of protein antigens). The *mxi-spa* system aids in exporting IpaB and IpaC to the bacterial surface so that the shigellae can rupture the wall of the endosome and escape into the host cell cytoplasm (Salyers & Whitt, 1994).

The formation of a tail of actin-myosin complexes allows the movement of the non-flagellated organism to the periphery of the cell. Movement in the cytoplasm is rapid and at random. A plasmid gene, *icsA* (also known as *virG*) is responsible for these events (Keusch, 2002). Lysis of the vesicle responsible for preventing the bacteria from entering the extracellular environment is dependent on a protein encoded by *icsB*.

An observation that many patients with *Shigella* or EIEC infections experience watery diarrhoea led to the suggestion that the organisms may elaborate secretogenic enterotoxins to bring about osmotic leaks of the mucosal epithelium. Nataro *et al.* (1995) identified one of the enterotoxins to be *Shigella* enterotoxin 2 (ShET2), which is encoded by the *sen* gene localized on the large virulence plasmids of 83% of *Shigella*

strains (including members of all four species) and 75% of EIEC strains. A deletion in the gene in EIEC strains had only caused decreased levels of enterotoxic activity. Purification of the *sen* gene product yielded a protein of a molecular mass of 63 kDa.

Other plasmid genes associated with virulence include *virF* gene whose product plays a central role in positive regulation of the plasmid virulence regulon. It directly activates transcription of the *icsA/virG* gene and indirectly activates *ipaABCD* (Sakai *et al.*, 1988). *virB* gene regulates the temperature regulation of *ipa* and *mxi-spa* expression (Maurelli *et al.*, 1984) and encodes a positive regulatory element that directs invasion gene expression (Tobe *et al.*, 1991). Expression of the gene itself is controlled by an additional positive regulatory element encoded by *virF*.

The virulence plasmid is able to integrate into the bacteria chromosome and silence the virulence genes. The silencing effect is caused by a failure in the expression of the *virB* gene whereby its promoter is placed in a DNA structural context that impedes its activation (Dorman & Porter, 1998). The plasmid maybe lost from the cell when the virulence gene regulon is induced. Schuch & Maurelli (1997) found that expression of the plasmid-encoded virulence regulon of *S. flexneri* 2a was induced by growth at 37^0C (i.e. the bacteria are phenotypically invasive), while growth at 30^0C repressed it, rendering the bacteria phenotypically noninvasive. In other words, virulence gene expression induces virulence plasmid instability.

1.4.2.2 Chromosomal-encoded

The pathogenetic capacity of *Shigella* is partially mediated by protein toxins encoded by chromosomal genes. *S. dysenteriae* type 1 produces a neurotoxic product called Shiga toxin, encoded by the iron-regulated gene *stx* (Keusch, 2002). It is among the most toxic biological agent known, including tetanus and botulinum type A toxins (Cantey, 1985). Non-*Shigella* organisms such as certain strains of *E. coli*, *Citrobacter*

freundii and *Enterobacter cloacae* are known to produce Shiga-like toxins (Sandvig, 2001). The family of Shiga toxins is under the AB group of protein toxins, i.e. toxins with one enzymatically active part (A) whilst the other part is bound to the cell surface (B). They kill cells by first binding to the cell surface, then they are endocytosed, and subsequently the A subunit enters the cytosol to inhibit protein synthesis and hence, causes a programmed cell death or apoptosis.

Another chromosomal protein toxin, *Shigella* enterotoxin 1 (ShET1) is almost exclusively elaborated by *S. flexneri* 2a (Noriega *et al.*, 1995); ShET1 has not been found in EIEC (Nataro *et al.*, 1995). Sequencing of the genes encoding ShET1 (*set1*) revealed two contiguous ORFs that encode proteins with a molecular size of 7 kDa (by *set1B*) and 20 kDa (by *set1A*). This discovery led to the speculation that the 55-kDa holotoxin derives from an A_1-B_5 configuration (Fasano *et al.*, 1995). Follow-up experiments confirmed the hypothesis that ShET1 was responsible for the watery phase of shigellosis. Fasano *et al.* (1997) reported that fluid accumulation in rabbit ileal loops caused by *S. flexneri* 2a was a time- and dose-dependent event in both the *in vitro* and *in vivo* rabbit animal model. The intestinal secretion was later found to be caused by the effect of ShET1 on inducible nitric oxide synthase (iNOS) expression to produce a significant increase in the concentration of nitric oxide (Rhee *et al.*, 2001). A higher nitric oxide concentration correlated to a larger amount of electrolyte secretion.

1.4.2.3 Pathogenicity islands

Pathogenicity islands (PAIs) are unstable regions on the chromosomes of certain pathogenic bacteria that are absent in the nonpathogenic strains of the same or closely related species, which contain large contiguous blocks of virulence genes (Hacker *et al.*, 1997; Maurelli *et al.*, 1998).

The tandem chromosomal genes *set1B* and *set 1A* are contained within a third oppositely orientated ORF, designated as *she*, in *S. flexneri* 2a (Noriega, 1995). In a later study, the three genes were found to be borne on a much larger 51-kb deletable chromosome element, which was referred to as the *she* PAI (Rajakumar *et al.*, 1997). The element was relatively unstable with a deletion rate of 10^{-5} to 10^{-6}. Nevertheless the instability of the newly discovered PAI may prove to be efficacious in lowering the potential reactogenicity of a *S. flexneri* 2a candidate vaccine strain, CVD 1205 by reducing the risk of undesirable horizontal gene transfer events (Mecsas & Strauss, 1996).

Another *Shigella* PAI was located in *S. flexneri* 5a and designated SHI-2 for *Shigella* island 2 (Moss *et al.*, 1999). It appeared to facilitate bacterial survival in stressful environments. Amongst the genes discovered in the island were genes encoding the aerobactin iron acquisition siderophore system and colicin V (ColV) immunity. The former allowed bacteria with SHI-2 to thrive in a low iron environment, while ColV immunity helped them to survive in competitive living space.

1.5 Laboratory diagnosis

1.5.1 Culture examination

Shigella are lactose nonfermenters, hence their colonies are often colourless on selective and differential agar plates. For the optimal isolation of the bacteria, two different selective media should be used: a general purpose low selectivity plating medium such as MacConkey, and a more selective agar medium like xylose lysine deoxycholate (XLD) agar (shigellae appear as pink-red colonies) or *Salmonella-Shigella* (*SS*) agar (Bopp *et al.*, 1998; Morello *et al.*, 1998). The identity of suspect colonies maybe screened biochemically or serologically. In fact, confirmation requires both biochemical and serologic identifications.

1.5.2 Biochemical identification

A second microbiological property used to screen for presumptive *Shigella* isolates is their inability to produce gas from sugars, a trait they do not share with *Salmonella*. Conventional screenings for this characteristic include inoculating the colony onto an agar medium with glucose and lactose, for example Triple sugar iron agar (TSI) or Kligler's iron agar (KIA). Shigellae characteristically produce an alkaline slant and an acid butt but do not produce gas or hydrogen sulphide. Additional biochemical tests are summarized in Table 1.1 (Bopp *et al.*, 1999; Keusch, 2002). Convenient commercial systems such as the API strips, which test for various biochemical properties, can only allow for a near-certain identification.

Table 1.1
Selected biochemical reactions of typical *Shigella* isolates (Bopp *et al.*, 1999; Keusch, 2002)

Biochemical test	Reaction
Oxidase	Negative
Motility	Negative
Acid from lactose	Negative (*S. sonnei* late positive)
Acid from glucose	Positive
Urease	Negative
Lysine decarboxylase	Negative
Ornithine decarboxylase	Negative (*S. sonnei* are positive)
Citrate	Negative
Methyl red	Positive
Voges-Proskauer	Negative

1.5.3 Serotyping

Although *S. dysenteriae* and *S. sonnei* are biochemically distinct, the remaining two species are often indistinguishable through biochemical screening. Therefore, serologic grouping is essential for the separation of *S. flexneri* and *S. boydii*, and is required for the confirmation of the other two species.

Serologic identification is typically performed by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed by, in some cases, testing with monovalent antisera for specific serotype identification. Due to the potentially serious nature of illness posed by *S. dysenteriae* type 1, isolates that agglutinate in serogroup A reagent must be sent to a reference laboratory promptly for further serotyping (Bopp *et al.*, 1999).

1.6 Polymerase chain reaction (PCR)

An essential principle of diagnostic microbiology is improvement of patient care by means of rapid detection and characterization of specific pathogens to ensure proper delivery of treatment. Over the past century, microorganism identification has relied heavily on phenotypic characteristics such as morphology and biochemical properties. Nonetheless, growth-based systems are usually time-consuming, expensive or simply not available.

Advancement of molecular biology especially in nucleic acid amplification technology has opened up new avenues for the detection, identification and characterization of pathogenic organisms in the clinical microbiology laboratories. First described in 1985 by its inventor Kary B. Mullis, polymerase chain reaction (PCR) has changed the way detection and characterization of nucleic acids are performed.

PCR is based on the ability of DNA polymerase to copy a strand of DNA as in the natural process of DNA replication. The enzyme initiates elongation at the 3' end of a short (primer) sequence band to a larger (target) strand of DNA. When two primers bind to complementary strands of target DNA, the sequence between the binding sites is amplified exponentially with each cycle of PCR.

1.6.1 The PCR process

Each PCR cycle consists of three steps (Figure 1.2).

1.6.1.1 DNA denaturation

Double-stranded target DNA is separated by heat (usually more than 90^0C) into two single strands. Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures; whereas, the bonds between deoxyribose and phosphates are stronger covalent bonds and they can remain intact.

1.6.1.2 Annealing of primer to target

Primers are short, synthetic sequences of single-stranded DNA typically made up of 20 to 30 bases and are specific for the target region of the organism. Two primers are included in the PCR: one for each of the complementary single DNA strands that is produced during denaturation. The beginning of the DNA target sequence of interest is marked by the primers which anneal (bind) to the complementary sequence. Annealing usually takes place between 40 and 65^0C, depending on the length and base sequence of the primers. This range of temperature allows the primer to anneal to the target sequence with high specificity.

1.6.1.3 Extension of target sequences

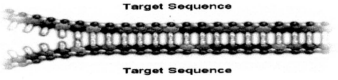
Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72^0C and the enzyme *Taq* DNA polymerase replicates the DNA strands. The enzyme is a recombinant thermostable DNA polymerase from the thermophilic bacterial species *Thermus aquaticus* (*Taq*). Unlike normal polymerase enzymes from other microbs, *T. aquaticus* strain YT1 is capable of growth at 70 to 75^0C and many of its enzymes are thermostable (Gelfand & White, 1990).

Taq DNA polymerase begins the synthesis process at the region marked by the primers. It synthesizes new double-stranded DNA molecules, both identical to the original double stranded target DNA region, by facilitating the binding and joining of the complementary nucleotides which are free in solution.

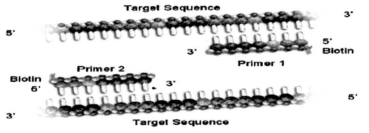
Extension always begins at the 3' end of the primer making a double strand out of each of the two single strands. *Taq* DNA polymerase synthesizes exclusively in the 5' to 3' direction. Therefore, free nucleotides in the solution are only added to the 3' end of the primers constructing the complementary strand of the targeted DNA sequence.

The DNA polymerase does not recognize the end of the sequence. The newly formed strands have a beginning, which is precisely defined by the 5' end of the primer, but the 3' end is not such defined. As the number of cycles increases, a strand with more defined length frequently serves as the template for the newly synthesized sequence. The DNA strand made from such a template has a precisely defined length which is limited at either end by the 5' end of each of the two primers. These DNA strands are called an amplicon.

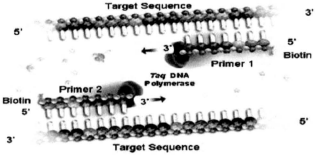
A PCR process takes place in a thermal cycler that automatically controls and alternates the temperatures for programmed periods of time for the appropriate number of PCR cycles (usually between 30 to 40 cycles). Generally, 30 to 50 thermal cycles result in a detectable amount of a target sequence originally presented in less than 100 copies (White *et al.*, 1992).



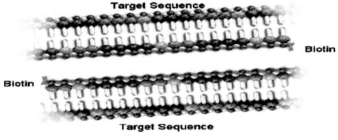
Step 1 – Denaturation by heat



Step 2 – Primer pair anneals to ends of target sequece



Step 3 – *Taq* DNA polymerase catalyses primer extension as complementary nucleotides are incorporated



End of first cycle – results in two copies of target sequence

Fig 1.2

Polymerase chain reaction (PCR)
(adapted from www.roche-diagnostics.com/ba_rmd/pcr_explained02.html)

1.6.2 Types of PCR-based amplification techniques

The original PCR process proposed in 1985 has undergone various modifications or used in conjunction with other detection techniques (Table 1.2).

Reverse transcription (RT)-PCR was developed to amplify RNA targets. In this process, RNA targets are first converted to complementary DNA (cDNA) by RT and are then amplified by PCR. Nested PCR uses two sets of amplification primers to increase sensitivity. One set is used for first round amplification that consists of 15 to 30 cycles. The products are then subjected to a second round of amplification with another set of primers specific for a sequence within the product of the first primers. Another variation is multiplex PCR, which will be discussed in depth in a later section. Arbitrarily primed PCR (AP-PCR) was developed in 1990 (Welsh & McClelland, 1990). It involves the use of a single 10- to 15-base, arbitrarily chosen primer to amplify genomic DNA under low-stringency conditions. Two isolates of the same species can be determined whether they are epidemiologically related.

Rapid thermal cycling for PCR amplification coupled with real-time fluorescence monitoring of the reactions (real-time PCR) has provided a new tool for rapid detection of microorganisms. The detection of PCR products is achieved by monitoring the change of fluorescence on the basis of fluorescence resonance energy transfer when the two hybridization probes anneal to the target DNA. As amplification and detection occur in the same reaction vessel and no post-amplification sample transfer is needed, the LightCycler platform reduces the risk of carryover.

Touchdown PCR was developed to increase the yield of specific products by lowering the annealing temperature one degree every second cycle to a lower limit or "touchdown" temperature. This touchdown temperature is then used for 10 cycles. Recently, this technique has been adapted for genomic sequencing to elucidate DNA sequences from known peptide sequences (Meisenholder, 1999). Long PCR enables

long stretches of DNA from 0.5 to 20 kb to be amplified. It is made possible with an enzyme mix containing both proofreading and polymerase activities to allow specific extension. As an alternative to restriction fragment length polymorphism (RFLP) analysis, it shortens the process from 6 to 8 weeks down to hours or days (Meisenholder, 1999).

Table 1.2
Examples of various PCR-based amplification techniques in the detection of microorganisms or specific genes

Technique	Organism(s)	Reference
RT-PCR	*E. coli*	McIngvale *et al.* (2002)
Nested PCR	*Vibrio vulnificus*	Lee *et al.* (1998)
Multiplex PCR	*Alloiococcus otitidis* *Haemophilus influenzae* *Moraxella catarrhalis* *Streptococcus pneumoniae*	Hendolin *et al.* (1997)
AP-PCR	*Legionella pneumophila*	Grattard *et al.* (1996)
Real-time PCR	*Toxoplasma gondii*	Lin *et al.* (2000)
Long PCR	Hepatitis A virus	Tellier *et al.* (1996)
Touchdown PCR	*Caenorhabditis elegans*	Lichtsteiner & Tjian (1993)
PCR-SSCP	*Triticum* (wheat)	Wang *et al.* (1997)
PCR-ELISA	*Campylobacter jejuni* *C. coli*	Sails *et al.* (2002)
PCR-immunomagnetic separation	*S. dysenteriae* type 1 *S. flexneri*	Islam & Lindberg (1992)

1.6.3 Applications of PCR in the study of *Shigella* spp.

PCR has helped tremendously in gaining more knowledge especially on the presence of *Shigella* in various types of environment and the dispersion of its different virulence-associated genes amongst shigellae isolates. Lampel *et al.* (1990) used it to detect enteroinvasive bacteria in foods by amplifying a 760-bp fragment within the large virulence plasmids carried by EIEC and *Shigella* spp. The sensitivity of PCR over conventional culture method was shown when Islam *et al.* (1998) discovered the 700-bp fragment of *ipaH* locus in 7 out of 23 culture-negative stools of dysentery patients in Bangladesh. Faruque *et al.* (2002) analyzed surface waters in Dhaka, Bangladesh for

the presence of shigellae, by a combination of PCR assays followed with concentration and culturing of PCR-positive samples. All the environmental shigellae were positive for the *ipaH* gene and all *S. dysenteriae* type 1 isolates had the *stx1* gene too. This environmental gene pool may contribute to the emergence of virulent *Shigella* strains especially amongst the population living near surface waters. Various species of *Shigella* were detected rapidly in environmental sewage by an immunocapture PCR with universal primers from conserved regions of the 16S rRNA sequence (Peng *et al.*, 2002). The method was more sensitive than the conventional universal primer PCR when it could detect the presence of 1 cfu of shigellae in 20 µl of cell suspensions. Bacteria in the environmental samples were detected without pre-treatment and test results were obtained within 5 hours.

1.7 Multiplex PCR

Multiplex PCR (mPCR) was first described in 1988 by Chamberlain *et al.* to simultaneously amplify multiple loci in the human dystrophin gene. The technique is a variant of PCR whereby two or more target sequences are co-amplified by including more than one primer sets in the same reaction. It may function as the end point of an analysis or it may be preliminary to further analyses such as sequencing or hybridization.

1.7.1 Reaction components

Basically, the reaction components of mPCRs are similar to single-locus PCRs. However, alterations in PCR buffer constituents, deoxynucleotides (dNTPs), magnesium chloride ($MgCl_2$) and enzyme concentrations in mPCRs greater than those required for most monoplex PCRs, usually result in considerable improvement in the sensitivity and/or specificity of the test (Markoulatos *et al.*, 2002).

1.7.1.1 Oligonucleotide primers

Primer selection follows simple rules such as a primer length of 18 to 24 bp, preferable with no complementary overlap between the paired primers at the 3' ends, the base composition of the primers should be as heterogenous as possible with an even mixture of all four bases and, a GC content of 35 to 60%. All the primer sets for a mPCR should have nearly the same melting temperature (T_m) or more specifically, temperature of annealing (T_a) to the template. The T_a can be calculated from the T_m by using the equation $T_a = T_m - 5^0C = [2(A+T) + 4(G+C)] - 5^0C$.

According to Chamberlain *et al.* (1990), the location of PCR priming sites needs to be chosen while considering the entire mPCR design. Flexibility in choosing the size of regions to be amplified aids in obtaining multiple reaction products that can be resolved on agarose gels. Different combinations of primers require different conditions for optimal multiplex amplification, and it will probably be necessary to optimize reaction conditions for any given set of oligonucleotides.

When the multiplex reaction is performed for the first time, equimolar primer concentrations of 0.1 to 0.5 μM are used. There will be uneven amplification with some of the products barely visible in the ethidium bromide-stained gels. Changing the proportions of various primers in the reaction usually does overcome this problem: amount of primers for the "weak" loci are increased while the "strong" loci's primers are decreased. The final concentration of primers may vary considerably among the loci and is established empirically. For low copy number or high-complexity DNA, the primer concentration should be 0.3 to 0.5 μM, and a 0.04 to 0.4 μM for high copy number or low-complexity DNA.

For optimum results, primers should be purified after synthesis by high-pressure liquid chromatography (HPLC) or gel purification. The primers should be stored at -20^0C when not in use, and aliquoted into smaller volumes as working solutions. The

shelf life of primers is at least six months in liquid form, and 12 to 24 months when stored dry after lyophilization (Atlas & Bej, 1994; Henegariu *et al.*, 1997; Markoulatos *et al.*, 2002).

1.7.1.2 PCR buffer

Henegariu *et al.* (1997) found that by increasing the buffer concentration to 2X or only the KCl concentration to 100 mM, mPCR worked with much better efficiency; more effective than adding adjuvants or PCR additives such dimethyl sulfoxide (DMSO), glycerol or bovine serum albumin (BSA). Generally, primer sets with longer amplification products are efficient at low salt concentrations, whereas higher salt concentrations suit primer pairs with short amplicons.

1.7.1.3 Magnesium chloride

Any PCR needs divalent magnesium ions. Although a recommended $MgCl_2$ concentration in a standard PCR is 1.5 mM at dNTP concentrations of around 200 µM each, by increasing the concentrations of $MgCl_2$ from 1.8 to 10.8 mM at a dNTP concentration of 200 µM each, unspecificity decreased and the products acquired comparable intensities at 10.8 mM (Henegariu *et al.*, 1997). Excessive magnesium ions stabilize the amplified double-stranded DNAs, thus preventing complete denaturation, which reduces yield. They can also stabilize spurious annealing of primers to incorrect template binding sites, decreasing specificity (Markoulatos *et al.*, 2002). Nevertheless, the optimal concentration will depend on the dNTP concentration, template DNA and PCR buffer composition.

1.7.1.4 Deoxynucleotides (dNTPs)

Free dNTPs are required for DNA synthesis. The four dNTPs (dATP, dTTP, dCTP and dGTP) should be used at equivalent concentrations to minimize misincorporation errors (Atlas & Bej, 1994). In a mPCR, best results of the amplification process were seen at concentrations of between 200 to 400 μM each dNTP value. Higher concentrations inhibited the working of the assay, whilst lower amounts produced amplified products barely visible on the stained agarose gel (Henegariu *et al.*, 1997). Primary stocks are usually diluted to 100 μM, aliquoted and stored at -20^{0}C. In fact, dNTPs are much more sensitive than *Taq* DNA polymerase to frequent cycles of thawing and freezing. After three to five cycles, mPCRs often did not work well (Henegariu *et al.*, 1997).

For efficient PCR reaction, the *Taq* DNA polymerase needs free magnesium ions (besides those bound by the dNTP and the DNA) (Innis & Gelfand, 1990). This maybe the reason why increases in the dNTP concentrations can rapidly inhibit mPCR but increases in magnesium ions often have positive effects.

1.7.1.5 *Taq* DNA polymerase concentration

The initial study that developed PCR utilized the Klenow fragment of *E. coli* DNA polymerase I to amplify specific targets from human genomic DNA (Saiki *et al.*, 1985). The inactivation of this polymerase at the high temperatures necessary for strand separation required the addition of enzyme after the denaturation step of each cycle. This laborious step was eliminated by the introduction of a thermostable DNA polymerase, the *Taq* DNA polymerase. Moreover, the upper size limit for amplification with the Klenow fragment was about 400 bp. Fragments as large as 10 kb have been synthesized with *Taq* and other thermostable enzymes (Erlich *et al.*, 1991).

The *Taq* DNA polymerase has no 3' to 5' exonuclease ("proofreading") activity but has a 5' to 3' exonuclease activity during polymerization, has an optimum activity around 70^{0}C and is not inactivated by short incubation periods at temperatures at which PCR-generated fragments will denature (90 to 95^{0}C). It has a half-life of approximately 45 min at 95^{0}C, hence exposure for this duration to temperatures more than 96^{0}C enhances faster enzyme inactivation and should be avoided (Atlas & Bej, 1994).

Recently, several archael DNA polymerases with proofreading ability have been commercialized for high-fidelity PCR. Of the enzymes characterized to date, *Pyrococcus furiosus* (*Pfu*) DNA polymerase exhibits the highest fidelity with an average error rate of 2- to 60-fold lower than other proofreading enzymes, and 6- to 100-fold lower than *Taq* (Hogrefe *et al.*, 2002). It was discovered that *Pfu* dUTPase improved the yield of products amplified with *Pfu* DNA polymerase by preventing dUTP incorporation and subsequent inhibition of the polymerase by dU-containing DNA.

Markoulatos *et al.* (2002) wrote that the most efficient enzyme concentration for mPCR seemed to be approximately 2.5U per 50 µl-reaction volume. Excessive enzyme caused an imbalance amplification of various loci and a slight increase in the background, possibly attributed to higher glycerol concentration in the stock solution.

To improve specificity of the reaction, at least one essential reagent, usually the enzyme, is withheld from the mixture until after the reaction reaches a temperature that favours specific primer annealing (Birch *et al.*, 1996). The "hot start" PCR is based on the fact that unspecific priming and production of unwanted amplicons are as result of the retention of considerable enzymatic activity at temperatures less than the optimum for DNA synthesis (Atlas & Bej, 1994). The use of different brands of thermally-activated DNA polymerases such as AmpliTaq Gold (Perkin-Elmer) has simplified the method. Heat restored the polymerase activity which was inactive at room temperature,

allowing the addition of a hot start to an existing PCR, with no changes to the protocol except the addition of a pre-PCR heat cycle (Birch *et al.*, 1996).

1.7.1.6 PCR additives

Additives such as DMSO, BSA or betaine have been reported to be beneficial in mPCRs (Elnifro *et al.*, 2000). The components may act to prevent the stalling of DNA polymerase, and also as destabilizing agents to reduce the T_ms of GC-rich sequences or as osmoprotectants by increasing the resistance of the enzyme to denature. On the other hand, in some studies (Henegariu *et al.*, 1997), DMSO and glycerol gave conflicting results. Therefore, the usefulness of these PCR additives needs to be evaluated individually.

1.7.1.7 DNA template

Amplification process needs at least one intact copy of the target gene, from less than 0.1 to a few kilobases. The total amount of DNA usually needed for PCR is 0.05 to 1.0 µg (Atlas & Bej, 1994). The lesser DNA used, the fewer unintended amplification products there will be. Amplification of single-copy genes is readily accomplished in 30 cycles from less than 0.2 µg of mammalian DNA. Genes present in higher copy numbers, for example mitochondrial DNA sequences, or in less complex genomes can be amplified from smaller quantities of template (Kocher & Wilson, 1991).

Many different methods are available for the isolation or extraction of DNA. Since PCR does not require highly purified template DNA, crude DNA preparations such as using a sterile toothpick to pick up bacterial colonies, diluting them in sterile dH_2O, boiling for 5 to 15 min followed by centrifugation to remove cellular debris, are often adequate. Moreover, slightly degraded or sheared DNA (as long as the target

sequence is intact) may amplify more readily because it denatures more easily, allowing better primer access to binding sites. Shorter templates also lower the probability of two non-specific priming events happening on the same template, hence lowering the amount of alternative amplicons (Kocher & Wilson, 1991). However, it is not recommended to store the crude lysates for a long period as their high levels of contaminants will result in DNA degradation.

For a preparation of high-purity DNA, organic solvents are used to extract contaminants from cell lysates (Sambrook & Russel, 2001). The cells are lysed using a detergent and mixed with phenol, chloroform and isoamyl alcohol. Correct salt concentration and pH must be used during extraction to ensure the contaminants are separated into the organic phase and DNA remains in the aqueous phase. DNA is then recovered by alcohol precipitation. Despite the high quality and stability of DNA extracted, the method is laborious and cumbersome. DNA molecules maybe contaminated with residual phenol and/or chloroform if removal of DNA from the aqueous phase is not properly performed.

Concurrently with the advancement in molecular biology, there are a wide variety of DNA extraction kits suited for a wide range of applications. QIAGEN's Dneasy Tissue Kits are designed for rapid isolation of pure total DNA (genomic, viral and mitochondrial) from various sample sources. They are highly suited for molecular techniques such as Southern blotting, PCR, real-time PCR and RFLP.

1.7.1.8 Oil overlay

A small amount of sterile mineral oil on top of the reaction mix prevents evaporation of the mixture during high temperatures and also helps to hasten the approach to the denaturation temperature. The overlay may maintain heat stability so

that salt concentrations remain unchanged during amplification processes in the thermal cycler (Atlas & Bej, 1994).

1.7.2 Reaction conditions

mPCR shares the same three steps in a cycle of amplification as other PCRs. It is performed by incubating the samples at three temperatures corresponding to those three steps, which make up a cycle of amplification.

1.7.2.1 Denaturation

According to Saiki (1989), one of the most common causes of failure in an amplification reaction is insufficient of heating during the denaturation step. To ensure a sufficiently high temperature for DNA strand separation to occur, a temperature range of 90 to 95^{0}C is often used, depending on the length and GC content of the fragment. Extended denaturation is unnecessary in order to maintain maximum polymerase activity throughout the reaction.

1.7.2.2 Annealing

The annealing temperature is one of the most important parameters in any PCR reaction. The ideal temperature is generally 5^{0}C below the true T_m of the primers. A temperature of 50 to 60^{0}C is enough for typical 20-base primers with 50% GC content. However, lowering of the annealing temperature by 4 to 6^{0}C is required for the same loci to be co-amplified in multiplex mixtures. When many specific loci are simultaneously amplified, the more efficiently amplified loci will negatively influence the yield of product from the less efficient ones. Long incubation at the required temperature is not required because it may lead to the production of nonspecific products (Saiki, 1989; Atlas & Bej, 1994; Henegariu *et al.*, 1997).

1.7.2.3 Extension

Primer extensions are typically performed at 72^0C. The incubation time at that temperature depends on the length of the fragment being amplified. Allowing one min for each kb of target is often enough. In mPCR, as more loci are amplified simultaneously, the pool of enzyme and dNTPs becomes a limiting factor. Thus, more time is necessary for the enzyme molecules to complete the synthesis of products. If the target sequence is 150 bases or less, this extension step can be eliminated altogether, and by using only two temperatures (for example, 55 to 75^0C for annealing and extension, 94^0C for denaturation), better PCR yields are seen (Saiki, 1989; Atlas & Bej, 1994; Henegariu *et al.*, 1997).

1.7.2.4 Number of cycles

The number of cycles necessary for an amplification process depends on the degree of amplification required and the need to amplify selectively the target sequence. Excessive cycles increase the amount and complexity of unspecific background products; insufficient number of cycles gives low product yield. Twenty cycles theoretically can produce one million copies of a sequence ($2^{20} = 10^6$). However, 28 to 30 cycles are usually good enough for a mPCR reaction (Atlas & Bej, 1994; Henegariu *et al.*, 1997).

1.7.2.5 Automation

Following the discovery of PCR, the three steps in each amplification were initially performed manually in heated water baths. Due to improper or inaccurate temperature regulation, PCR results were irreproducible. Since the introduction of the first DNA thermal cycler by Perkin-Elmer Cetus Corp. (Atlas & Bej, 1994), different designs of the machine have been manufactured.

It is important that a thermal cycler provides uniform heating-cooling cycles consistently in all reaction wells and produces a profile in the sample tube similar to the cycle that has been programmed. Each reaction well must be able to give precise temperature so as to provide the required stringency for primer annealing, accurate conditions for strand dissociation and extension (Atlas & Bej, 1994). Existing models in today's market have efficient thermal controls such as Peltier pump, light, film heater, electric coil or air and robotic hot block to heat up or cool down metal blocks (Meisenholder, 1999).

The newer generation of automated machines is faster than its predecessor, utilizes thin-walled plastic tubes to facilitate heat transfer and can accommodate more samples. Some of the thermal cyclers have been installed with programmable temperature gradient profiles for easier optimization processes (Erlich *et al.*, 1991).

1.7.3 Contamination of mPCR

The most common problem in any amplification reaction is contamination. Amongst the sources of nucleic acid contamination are products of a previous PCR reaction ("product carryover") accumulated in the laboratory as a result of repeated amplification of the same target sequence, exogenous DNA or other cellular materials in PCR reagents, and cross-contamination between clinical specimens.

Several approaches listed below to minimize false positives in PCR are often practiced in the laboratory (Kwok & Higuchi, 1989):

a) Physical separation of pre- and post-PCR activities

Reactions should be set up in a separate room or containment unit such as a biosafety cabinet with ultraviolet germicidal lamps to damage any leftover DNAs. A separate set of supplies and dedicated pipettors should be kept in this area and be used only for setting up reactions. Amplified DNA must not be brought into this area.

Analyses of PCR amplicons are carried out in another working space with a different set of supplies and pipettors.

b) Positive displacement pipettors

In order to prevent barrel of pipetting devices being contaminated with aerosols of sample DNA, positive displacement pipettors with disposable tips and plungers are recommended for use. Newer generations of pipettors have removable parts which can be autoclaved to ensure sterility.

c) Autoclave PCR solutions and supplies

Buffer solutions, deionized water, disposable pipette tips and microcentrifuge tubes can be autoclaved without comprising on their performance. Autoclaving under conditions that provide bacterial decontamination degrades DNA to a very low molecular weight. Primers, dNTPs and the enzyme, however, cannot be autoclaved.

d) Aliquot reagents

It is advisable to divide reagents into aliquots of smaller volume to minimize the number of repeated samplings. Preparation, aliquotting and storage of the reagents must be performed in a PCR-amplicon free area.

e) Substitution of dUTP for dTTP

To distinguish PCR products from template DNA, dUTP is substituted for dTTP in the assay and is incorporated into the amplification products. The presence of this unconventional nucleotide allows the distinction of products of previous PCR amplifications form the native DNA of the sample. The enzyme uracil N-glycosylase (UNG) that is present in the reaction pre-mix, will catalyze the excision of uracil from any single- or double-stranded PCR carry-over DNA present in the reaction prior to the first PCR cycle (Erlich *et al.*, 1991).

f) Short-wavelength ultraviolet irradiation

Reaction mixtures without the presence of DNA templates are sterilized with UV light. Sarkar & Sommer (1990) revealed that irradiation of the PCR mixture did not compromise the ability of the reagents to mediate efficient amplification. Irradiation with both 254- and 300-nm UV lights was more effective than the single usage of each light. Opposedly, Cimino *et al.* (1990) cautioned against applying direct UV irradiation as a sterilization process in a pre-amplification mode, whereby both the length of the PCR product and its internal sequence must be considered.

g) Meticulous laboratory techniques

Additional precautions that should be considered include:

(i) wearing and changing non-powdered disposable gloves when entering or reentering different areas when PCR work is performed,

(ii) spinning down any liquid from the sides and top of the closed tube before opening it,

(iii) uncapping and closing tubes carefully to prevent aerosols, and

(iv) adding sample DNA last after pipetting in buffer, primers, enzyme, dNTPs, deionized water and mineral oil into the reaction tubes, and capping each tube before proceeding to the next tube.

h) Judicious selection of controls

A sample, which amplifies consistently should be selected as a positive control to avoid false results. Every PCR assay must have a reagent control containing all the necessary components for amplification except DNA template, to check for the presence of minute number of PCR products in the reagents that may lead to sporadic positive results.

1.7.4 Applications

Numerous multiplex PCRs have been applied for the simultaneous detection of pathogenic microorganisms as well as specific genes (Table 1.3).

Table 1.3
Examples of mPCR applications

Organism	Quantity of primer sets	Publication
Aeromonas hydrophila *S. flexneri* *Yersinia enterocolitca* *Salmonella typhimurium* *V. cholerae* *V. parahaemolyticus*	6	Kong *et al.* (2002)
Listeria spp.	5	Bubert *et al.* (1999)
Salmonella enterica	5	Hirose *et al.* (2002)
Candida spp. *Aspergillus fumigatus* *Cryptococcus neoformans*	5	Luo & Mitchell (2002)
Enterotoxigenic *E. coli* *Shigella* spp.	3	Frankel *et al.* (1989)
Shigella spp.	3	Houng *et al.* (1997)
Leishmania spp.	3	Harris *et al.* (1998)
Salmonella spp. *Shigella* spp.	2	Vantarakis *et al.* (2000)

1.8 Treatment and prevention

1.8.1 Treatment

In shigellosis, the onset of dysentery is often preceded by an 18- to 24-hour phase of watery diarrhoea. At this juncture, the most important supportive therapy is control of dehydration via an oral rehydration solution (ORS). Yet, in the management

of most symptomatic *Shigella* infections, ORS-appropriate antimicrobial agent remains the therapy of choice.

Increased incidences of resistance to antimicrobial agents among *Shigella* spp. present a major threat in the treatment of shigellosis. The difficulty in recommending a drug of choice is also confounded by the shift in the prevalent serogroups and changing pattern of antimicrobial susceptibilities amongst the isolates. For example, in Calcutta, shigellae strains from January to December 1997 were found to be resistant to cotrimoxazole, tetracycline and furazolidone with an increased trend of development of resistance to nalidixic acid (Niyogi *et al.*, 2000). In Barcelona, high rates of resistance towards trimethoprim-sulfamethoxazole, ampicillin and chloramphenicol existed in the 1995 to 1998 period (Prats *et al.*, 2000). Consequently, antimicrobial susceptibility testing is done to determine the most effective agent for individual strains.

Generally, fluoroquinolones and pivamdinocillin are effective for treating multidrug-resistant (MDR) *Shigella* infections. Fluoroquinolones such as ofloxacin, norfloxacin and ciprofloxacin are not recommended for treating children due to their toxicity. Newer drugs, for example the oral form of the extended-spectrum cephalosporin cefixime was active in vitro against MDR shigellae but failed clinically due in part to poor intracellular activity of the cephalosporins (Salam *et al.*, 1995).

Recently, a macrolide agent azithromycin used primarily to treat Gram-positive infections, was evaluated for its efficacy in shigellosis treatment. In the treatment of adult men with moderate to severe shigellosis, it achieved an 82% clinical rate of cure. It has a low incidence of adverse effects and is safe in children. Development of resistance to agents currently in use should not result in resistance to this drug as it belongs to a different class of microbial agents from the current drugs used to treat shigellosis (Khan *et al.*, 1997).

1.8.2 Prevention

Shigellosis can be prevented with appropriate control of its human reservoir and sanitary control of its environmental sources via water chlorination, having adequate sewage disposal, fly control, and protection of food, water and milk from human or mechanical vectors. Immunity to *Shigella* is species as well as serotype specific (Levine, 2000). With the observation that antibodies directed against the lipopolysaccharide O antigen largely mediate immunity, trials of conjugate vaccines had been performed. A parenteral vaccine of *S. sonnei* O polysaccharide linked to *P. aeruginosa* exotoxin A was found to be well tolerated and highly immunogenic in Israeli soldiers, with a single dose of the vaccine conferring 74% protection against *S. sonnei* infection (Cohen *et al.*, 1997).

Alternatively, attenuated shigellae can serve as safe and protective live oral vaccines. A live attenuated *S. flexneri* 2a vaccine candidate, CVD 1207, had specific deletions in *virG*, *sen*, *set* and *guaBA* (Kotloff *et al.*, 2000). With these engineered deletions, the strain was still able to invade but could not spread intercellularly due to the deletion of *virG*, there was no enterotoxin production (Δsen and Δset) and had limited proliferation *in vivo* ($\Delta guaBA$). CVD 1207 was reportedly well-tolerated at as high as 10^8 cfu. There were only a few subjects who suffered mild diarrhoea due to the highest dosages (10^9 to 10^{10} cfu), otherwise no subject had fever or dysentery.

To date, almost all the leading strategies to develop vaccines to prevent diarrhoeal disease and dysentery attributed to *Shigella* are still in various phases of clinical trials. Therefore, preventive steps remain the most efficient method against the infections.

1.9 Rationale for study

Isolating an invasive bacterial pathogen such as *Shigella* from faeces, is about the only method to determine with certainty that an episode of dysentery is brought by a specific bacterium agent. Nevertheless, these invasive bacteria often require special culture media and unusual growth conditions to grow and to be identified morphologically.

Confirmation of the identity of the isolate(s) is performed by slide agglutination tests with specific diagnostic antisera that are often not available widely in developing countries. Moreover, results of these routine tests are only available after two or three days. Attempts to isolate *Shigella* from faecal specimens encounter failure as the pathogen seldom survives pH changes that occur in specimens, which are not properly and timely delivered to the laboratory even if refrigerated. With the accessibility to drugs for treatment, there is a pressing need for rapid and specific diagnosis especially for the institution of appropriate therapy to old, young and immunocompromised individuals.

Although there are numerous publications on the use of PCR to detect for shigellae in clinical samples, those studies concentrated mainly on the detection of a single gene: chromosomal or plasmid-encoded genes, both of which are known to be unstable. The design of a mPCR presented in this thesis incorporates the detection of both groups of virulence-associated genes, thus lessening the rate of false negatives due to gene deletions.

1.10 Objectives of study

The objectives of this study are:

a) to compare the efficiency of two DNA extraction methods: boiling and phenol-chloroform extraction,

b) to establish the reproducibility and specificity of both PCR assays: multiplex PCR and monoplex PCR,

c) to test the sensitivity of multiplex PCR in bacterial culture and spiked faecal samples,

d) to determine the prevalence of five virulence-associated genes (*set1A*, *set1B*, *ial*, *ipaH* and *sen*) in Malaysian strains of *Shigella* spp. isolated between years 1997 to 2000, by using PCRs, and

e) to compare the efficacy of multiplex PCR and microbiological method in clinical specimens.